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## **Introduction and Overall Objectives**

After traumatic brain injury (TBI), the human brain sometimes develops tau pathology partly resembling the tauopathy that is well-established as a hallmark neuropathological feature of Alzheimer's disease (AD). In healthy brain cells, tau is a component of the microtubule network with vital roles in cytoskeletal structure and intracellular transport. However, within vulnerable neurons in signature regions of the AD brain, tau becomes hyperphosphorylated, dissociated from microtubules, aggregated, and mislocalized within cell bodies and proximal dendrites instead of axonal processes, abnormalities that collectively are referred to as tauopathy. There is considerable evidence in AD that tauopathy drives the loss of neurons and synapses underlying the onset and progression of regional brain atrophy and cognitive impairment. Given that AD is a slowly progressive neurodegenerative and cognitive disorder, and TBI induced by inertial forces, concussive blows, or blast will sometimes lead to chronic, progressive brain atrophy and cognitive decline, the question arises whether AD and TBI may share common underlying tau-dependent pathophysiology. At present, although tau is known to accumulate after TBI and become phosphorylated on multiple residues, its pathophysiological importance to brain damage and dysfunction during the acute and chronic post-injury time periods is unknown. From human TBI studies, it is difficult to determine the contribution of tau to progressive brain damage and dysfunction, owing to their dependence on non-invasive or post-mortem histopathological methods. Furthermore, there are currently no simple, validated blood tests for diagnosing at an early and potentially treatable stage the subset of TBI sufferers that go on to develop chronic neurodegenerative disease and progressive cognitive impairment.

Previously, we established a new translational mouse model for studying pathogenic mechanisms of tau, using a viral vector to drive robust long-lasting expression of a pathological form of human tau focally within a specific hippocampal input pathway that is both preferentially vulnerable in early-stage AD and critically important for long-term memory. The model confines expression of mutant human tau to the lateral perforant pathway, the projection from the lateral entorhinal cortex to the hippocampal dentate gyrus. This mouse model of early-stage AD tauopathy is characterized by rapid, dose-related, circumscribed human tau expression, tauopathy, trans-synaptic spread of human tau expression, and tau-dependent neurodegeneration. The model is exceptionally well suited for addressing whether human tau affects structure and function of the hippocampus after single or repetitive mild TBI, and whether mild TBI exacerbates ongoing tauopathy to promote a chronic neurodegenerative condition. In addition, over the past decade we have discovered and characterized new biofluid-based markers for neurodegeneration. Whereas these novel biomarkers have shown considerable promise as diagnostic and prognostic tools in acute trauma- and ischemia-induced brain injuries, they have never been evaluated as a potential blood diagnostic test for a TBI-associated chronic neurodegenerative condition.

## Objectives

To study tau/TBI interactions and validate preclinically new biofluid-based diagnostic markers for chronic TBI-induced progressive brain atrophy and cognitive decline, we are collaborating with Dr. Douglas Smith, Director of the Penn Center for Brain Injury and Repair, who pioneered the development and characterization of a controlled cortical impact (CCI) model of TBI in the mouse, and our mutual colleague Dr. Victoria Johnson. We combine mild CCI with our novel mouse model of early-stage AD tauopathy to study three critical unanswered questions: (1) does tau exacerbate the acute and chronic effects of mild TBI on brain structure

and function? (2) does mild TBI worsen an ongoing tauopathy to promote development of a chronic neurodegenerative disorder? (3) do blood levels of biomarkers for neurodegeneration diagnose the subset of mild TBI cases developing acute brain damage, or chronic progressive neurodegeneration and behavioral dysfunction?

Our first two objectives address whether tau is an important target for therapeutic intervention in TBI. Our third objective is to discover and validate pre-clinically a blood test for improving the diagnosis of TBI-induced chronic neurodegenerative disease in the long-term post-injury time period.

### **Keywords**

Tauopathy; tau phosphorylation; traumatic brain injury; concussion; neurodegeneration; entorhinal cortex; perforant pathway; synapse loss; cognitive dysfunction; prognostic biomarker; diagnostic marker; brain atrophy; chronic traumatic encephalopathy; Alzheimer's disease.

## Accomplishments through Year 2

### Highlights from Year 1

- Used a novel mouse model of early-stage Alzheimer-type tauopathy, with hippocampal input-specific expression of human tau P301L, to evaluate interactions between pathological human tau and controlled cortical impact mild TBI (all 3 objectives)
- Generated evidence that, compared with eGFP as a control protein, pathological human tau does not appreciably change the vulnerability of neurons, axons, and synapses of this hippocampal input to a single mild TBI in the acute post-injury period (objective 1)
- Generated evidence that single mild TBI does not appreciably change the expression level, distribution, or phosphorylation of human tau at 7 days post-injury (objective 2)
- Generated evidence that single mild TBI impairs hippocampus-dependent spatial learning acutely post-injury, and pathological human tau does not exacerbate this cognitive dysfunction (objective 1)
- Developed a serum sample bank from mice subjected to either sham injury or single mTBI and characterized for cognitive and histopathological changes acutely post-injury, for pending analysis of candidate diagnostic and prognostic surrogate markers for TBI-induced brain damage and dysfunction (objective 3).

### Highlights from Year 2

- Completed all structural, functional, and biomarker analyses of interactions between pathological human tau and both single and double mild TBI during the acute post injury period (milestone 1)
- Demonstrated that pathological human tau expressed in a specific hippocampal input, the lateral perforant pathway, does not endanger the pathway to either single or repetitive mild TBI acutely (within 7 days) post-injury
- Demonstrated that neither single nor repetitive mild TBI appreciably change the development or distribution of tau pathology in the pathway acutely post-injury
- Demonstrated that pathological human tau expressed unilaterally in the pathway does not cause a spatial learning deficit by itself, and does not worsen the subtle spatial learning deficit elicited by single and repetitive mild TBI
- Demonstrated that serum levels of SNTF, a mechanism-based biomarker for injury-induced axonal damage, are not changed appreciably after single or repetitive mild TBI at 7 days post-injury, or affected by pathway-specific expression of pathological human tau
- Launched the second phase of the project, focused on interactions between tauopathy and mild TBI in the long-term post-injury period; thus far, 60 mice have been genetically modified and subjected to single or double mild TBI, or to sham injury; they now await qualitative and quantitative analyses of the effects of pathological tau on hippocampal structural, functional, and blood biomarker long-term changes after mild TBI.

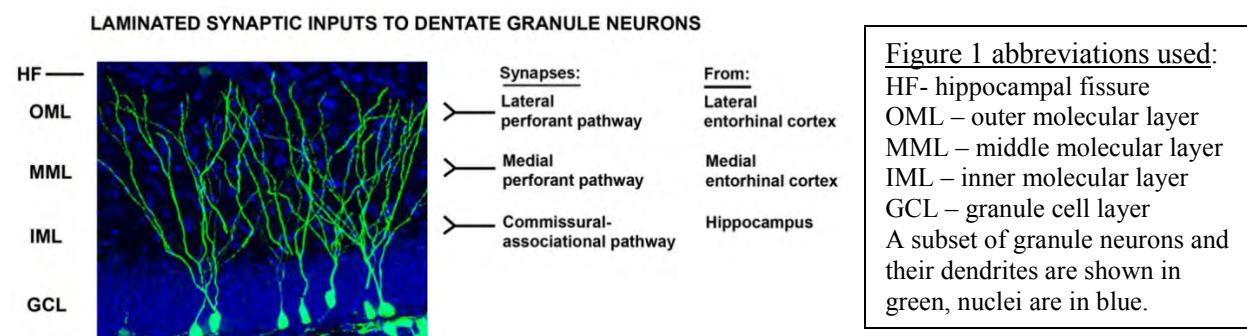
#### 1. The novel mouse model of early-stage Alzheimer-type tauopathy

There is considerable evidence that dysfunction of the perforant pathway projection from entorhinal cortex (EC) to hippocampal dentate gyrus is an important contributor to the onset and progression of cognitive impairment in AD. This pathway is a major source for excitatory innervation of hippocampus, a structure vital for memory formation. Damage to the EC or perforant pathway projection in animals causes a rapid forgetting syndrome reminiscent of AD. The perforant pathway is especially vulnerable in AD. The entorhinal neurons of origin in layer

II are among the first to develop aggregates of hyperphosphorylated tau in the form of neurofibrillary tangles (Braak stage I), and the terminal field in the dentate gyrus is a preferential early site for amyloid A $\beta$  deposition. Recent evidence suggests that tauopathy initiating in the perforant pathway spreads over time through its afferent connections. Finally, the pathway dies beginning with the earliest signs of cognitive impairment, and the neuronal loss progresses coincident with cognitive decline, until more than 90% of the pathway has degenerated.

Consequently, we used an AAV vector approach to express pathological human mutant tauP301L linked genetically to human tauopathies or an eGFP control focally in the mouse lateral perforant pathway. The vectors are microinjected by unilateral stereotaxic convection-enhanced delivery into the right lateral EC, and four weeks are allowed for the foreign proteins to be expressed in the entorhinal layer II neurons of origin, the perforant pathway axons, and their synapses onto the distal dendrites of granule neurons in the dentate gyrus outer molecular layer (OML). As shown schematically in Figure 1, in the mouse all of the synaptic inputs terminating in the OML originate from the lateral EC and lateral perforant pathway projection.

Confirming our earlier publications (J Neuropathol Exp Neurol 72: 1062-1071 [2013]; PLoS One 10: e0142340 [2015]), delivery of AAV-hTauP301L to the right lateral EC leads within 3-4 weeks to robust human tau expression in the lateral perforant pathway layer II neurons of origin and the entire lateral perforant pathway projection as it traverses the stratum lacunosum-moleculare (SLM) of hippocampal CA1 sector before perforating the hippocampal fissure (HF) to terminate in the dentate gyrus OML (see Annual Report, 2015). The human tau is distinguished from the widely distributed endogenous mouse protein by immunohistochemistry using the human specific monoclonal HT7. In contrast, tau phosphorylated on serine residues 202 and 205, considered an early marker for hyperphosphorylation and aggregation and labeled with the monoclonal AT8, is confined to layer II neurons of the lateral EC, but does not undergo appreciable axonal transport and is below the limit of detection in the perforant pathway axons or synapses. This distribution pattern of IAT\*-labelled human tau closely resembles the earliest neuropathological stage of Alzheimer-type tauopathy (Braak stage I) with hyperphosphorylated, aggregated tau localized to the superficial trans-entorhinal region. An identical distribution pattern is observed for tau phosphorylated on either Thr231 or Ser262. After delivery of AAV-eGFP as a control, the autofluorescent eGFP protein distributes similarly to pathological human tau (hTau) throughout the lateral perforant pathway neurons of origin, axons, and pre-synaptic terminals innervating the hippocampal dentate gyrus and terminating in the OML.



## 2. Study questions, and numbers of mice evaluated so far

In its first 18 months, our project focused on milestone 1, addressing three key questions in the search for tau/mTBI interactions in the acute post-injury period:

- (1) What is the effect of pathological hTau on the acute response to mTBI?
- (2) Does mTBI exacerbate tau pathology or promote its anatomical spread acutely post-injury?
- (3) Is there a blood biomarker for neurodegeneration that at 7 days post-injury serves as a surrogate marker for acute mTBI-induced brain damage and dysfunction?

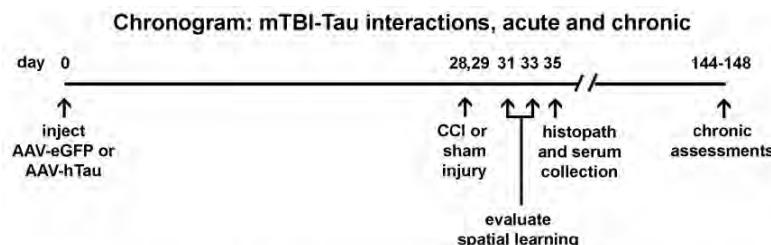
For this work, we analyzed the following groups of mice in the short-term post-injury period:

- 1) AAV-eGFP, sham injury (n=10)
- 2) AAV-hTau, sham injury (n=15)
- 3) AAV-eGFP, mild TBI (n=11)
- 4) AAV-hTau, mild TBI (n=9)
- 5) AAV-eGFP, double mild TBI (n=9)
- 6) AAV-hTau, double mild TBI (n=10)

Our work plan detailed in the year one Annual Report was completed in year two of the project. In total, we performed 15 AAV microinjections per group, with the expectation that approximately 70% of the vector microinjections would be successfully placed in the right lateral entorhinal cortex and drive robust foreign protein expression throughout the entire extent of the lateral perforant pathway. This experimental design yielded sufficient numbers of each experimental group for drawing definitive conclusions on all three study questions posed above. Of the injected mice, 64 across the 6 treatment groups were confirmed by blinded histological analysis to have received well placed AAV vector microinjections and exhibit strong eGFP or hTau transgene expression throughout the entire rostro-caudal extent of the lateral perforant pathway. At one month after gene delivery, the mice were injured and then evaluated for the following hippocampal structural (at day 7) and functional (at days 3-5) endpoints:

- (a) human tau expression (human-specific tau antibody HT7)
- (b) tau phosphorylation (pTau202/205 antibody AT8; pTau231 antibody PHF6)
- (c) human tau trans-synaptic propagation (human-specific tau antibody HT7)
- (d) perforant pathway neuronal survival (NeuN antibody)
- (e) perforant pathway synapse and axonal integrity (synaptic zinc using Timm's stain)
- (f) perforant pathway axonal pathology (APP and SNTF antibodies)
- (g) hippocampus-dependent spatial learning (Morris Water Maze on days 3-5)

In addition, serum samples were obtained at the time of sacrifice on day 7 post-injury for quantification of the calpain-derived spectrin fragment SNTF (nonerythroid  $\alpha$ -spectrin 1-1176) as a blood biomarker for mTBI. This past year, we completed all of the behavioral, histological (both qualitative and quantitative), and serum biomarker analyses for the mice with well-place vector injections and robust lateral perforant pathway transgene expression. This completed milestone 1 of the project. The following sections describe our findings and summarize conclusions on the influence of pathological hTau on the short-term outcomes from single and double mTBI, and the short-term influence of single and double mTBI on the severity and distribution of tau abnormalities in a novel mouse model of early-stage Alzheimer tauopathy.



**(1) A mild controlled cortical impact TBI in the mouse elicits subtle hippocampal structural damage on the impacted side.**

One of our objectives is to examine acute and chronic effects of mTBI on the structural and functional integrity of a major hippocampal input pathway when it expresses a pathological form of human tau. Consequently, it is important that the chosen method for inducing mTBI elicits discernable but mild hippocampal structural damage in the absence of expression of pathological tau. To accomplish this, the magnitude of the controlled cortical impact was adjusted by varying the impounder velocity and cortical depth, so as to produce minor but readily detectable hippocampal structural damage at 7 days post-injury. As shown in Figure 2, beneath the impacted lesioned parietal cortex (middle panel, asterisks), the hippocampus is largely intact. However, three overt changes to hippocampal structure occur acutely post-injury. First, the ipsilateral hippocampus exhibits localized swelling and disrupted cytoarchitecture in the CA1 sector near the CA2/3 border (arrow) compared with the uninjured contralateral side. Secondly, there is compression of the dentate gyrus. Compared with the uninjured contralateral region, the thickness of the dentate outer molecular layer is reduced 10% by single mTBI and 12% by double mTBI. The disrupted cytoarchitecture and compression of the dentate outer molecular layer are observed for injured mice expressing both eGFP and hTauP301L in the lateral perforant pathway afferents and terminal field. Finally, immunostaining for SNTF, which marks necrotic neurodegeneration, reveals scattered mTBI-induced acute degeneration of hippocampal CA1 neurons beneath the site of controlled cortical impact at 7 days post-injury (right panel, arrows), and this, too, occurs irrespective of the foreign protein expressed in the lateral perforant pathway.

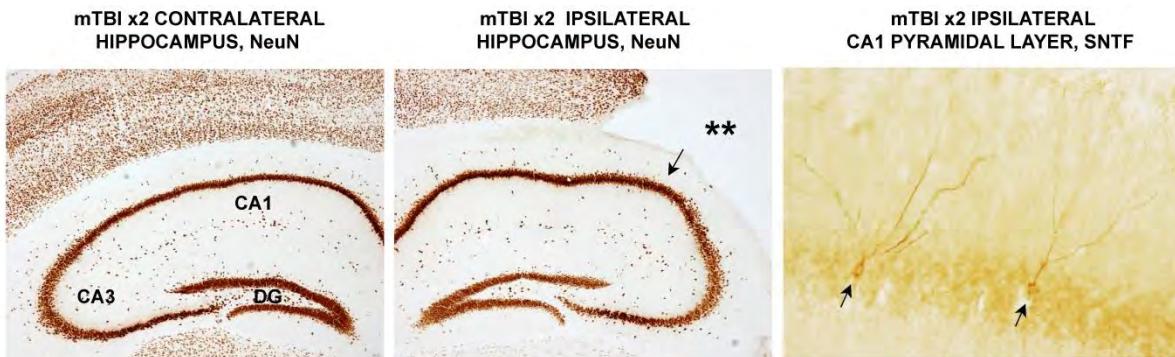


Figure 2. Mild hippocampal structural damage with repetitive controlled cortical impact TBI ipsilateral to the genetically modified lateral perforant pathway. Left and middle panels: neuronal nuclei stained for NeuN at 7 days post-mTBI; the double asterisk denotes the lesion in parietal cortex at the site of the controlled cortical impact. The arrow identifies a zone of disrupted cytoarchitecture in the hippocampal CA1 sector. Right panel: immunostaining for SNTF, which marks neurons acutely undergoing necrotic neurodegeneration (Siman et al., J. Neurosci. 14, 3934 [1994]; Saatman et al., J. Neuropathol. Exp. Neurol. 55, 850 [1996]). The arrows identify scattered acutely degenerating hippocampal CA1 pyramidal neurons at 7 days post-injury. These changes in hippocampal structure are not observed in sham-injured mice, confirming that they result from mTBI. See 2015 Annual Report for additional detail on the structural effects of single mTBI.

**(2) Expression of eGFP as a control does not promote degeneration of perforant pathway neurons, axons, and synapses after TBI.**

As described in prior reports and substantiated over the past year, at 7 days after either single or double mTBI, zinc staining of lateral perforant pathway synapses in the dentate outer

molecular layer is unchanged in the eGFP control group. Despite strong expression of eGFP in the lateral perforant pathway axons in the hippocampal SLM and synaptic field in the dentate outer molecular layer, there is no change in presynaptic terminal density or thickness of the layer. Similarly, at 7 days post-injury there is no overt loss of lateral perforant pathway neurons in layer II of the lateral entorhinal cortex expressing the eGFP control in comparison to the uninjured contralateral side, based on qualitative and quantitative analyses of NeuN-immunopositive neuronal nuclei. These data established our readiness to determine whether expression of human tauP301L in the pathway in place of eGFP endangers these neurons and synapses to the acute effects of mTBI.

**(3) Expression of human tau below the threshold level for direct neurotoxicity does not enhance perforant pathway neuronal vulnerability to single or repetitive mTBI in the acute post-injury period.**

Injury-induced neuronal loss in the lateral entorhinal cortex was assessed in the presence of pathological human tau expression following NeuN immunostaining of neuronal nuclei. As shown by quantitative analysis in Table 1, for mice expressing eGFP in the right lateral perforant pathway as a control foreign protein, neither single nor double mTBI caused appreciable damage to the lateral perforant pathway neurons of origin in layer II of the lateral entorhinal cortex. Identical results were obtained for mice expressing hTauP301L in the lateral entorhinal cortex and perforant pathway projection (Figure 3, bottom panels, and Table 1). Our results demonstrate that, in the acute post-injury period (7 days), pathological human tau does not endanger perforant pathway neurons to degeneration induced by single or double mTBI.

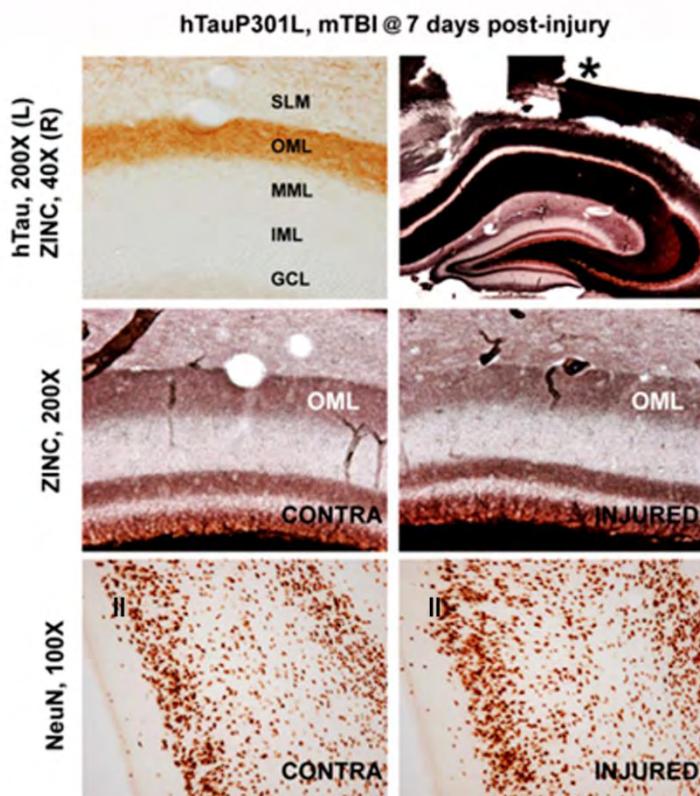


Figure 3. mTBI does not damage lateral perforant pathway neurons expressing pathological hTau at 7 days post-injury, and has little effect on survival of axons and synapses.  
 Top left - hTau expression persists in the lateral perforant pathway synaptic zone in the dentate OML at 7 days after mTBI. (200X mag).  
 Top right - the mTBI has a minor effect on hippocampal structure beneath the impact site (asterisk). (Zinc staining, 40X mag)  
 Middle panels - Staining for pre-synaptic vesicle zinc in the lateral perforant pathway terminal field in the OML. Note that there is little appreciable difference in synaptic density between the uninjured side and the hTau-expressing injured side, despite the disrupted cytoarchitecture of the dentate gyrus. (200X mag).  
 Bottom panels - Neuronal staining with NeuN shows that mTBI coupled with hTau expression does not cause appreciable loss of lateral perforant pathway neurons originating in layer II of the lateral EC. (100X mag).

**Table 1.** Quantitative morphometric analysis of the effects of mTBI on lateral perforant pathway neuronal survival: pathological human tau combined with mTBI does not trigger neuronal loss. Survival of lateral perforant pathway neurons is represented as the mean NeuN positive density of layer II neurons on the injured side relative to the control hemisphere for each brain section.

Group	N	Injured (%contra +/- sem)	P value (unpaired t-test)
eGFP sham	10	96.5 +/- 2.0	
eGFP mTBI	9	92.7 +/- 2.9	0.3 vs eGFP sham
eGFP mTBI x2	9	100.8 +/- 3.5	0.3 vs eGFP sham
hTau sham	9	102.9 +/- 4.5	0.2 vs eGFP sham
hTau mTBI	5	91.0 +/- 6.7	0.2 vs tau sham; 0.8 vs eGFP mTBI
hTau mTBI x2	9	93.6 +/- 3.9	0.15 vs tau sham; 0.2 vs eGFP mTBI x2

#### (4) Synapse integrity after mTBI: lack of acute effect of pathological human tau.

In the hippocampus, staining for pre-synaptic terminal zinc is a pathway-specific method for analyzing the integrity of afferent inputs arising from multiple brain regions, including from the lateral entorhinal cortex via the lateral perforant pathway. During the past year, we completed quantitative analysis of synapse survival at 7 days after single and double mTBI in comparison to sham injury for all mice expressing either eGFP or hTauP301L in the pathway. For each mouse, 3 sections were analyzed by comparing zinc staining intensity in the injured dentate outer molecular layer as a function of the staining intensity of the comparable uninjured synaptic field within each brain section. As shown in Figure 3 (above, middle panels) and Table 2, for mice expressing eGFP- or hTau in the right lateral perforant pathway, neither single nor double mTBI triggered a loss of lateral perforant pathway synapses. Our prior work establishes that the method is readily capable of detecting even minor loss (<30%) of lateral perforant pathway synapses (PLoS One 10: e0142340 [2015]; see also Section 5 below). We conclude that neither single nor double controlled cortical impact mTBI damages the lateral perforant pathway axons or synapses, and expression of hTauP301L does not endanger the pathway acutely to mTBI. This conclusion is substantiated by immunostaining for axonal damage after TBI (e.g., Johnson et al., Acta Neuropathol. 131, 115 [2016]). Neither APP nor SNTF immunohistochemistry at 7 days post-injury reveals axonal pathology in the perforant pathway afferents in the stratum lacunosum-moleculare (SLM) of the CA1 sector, or in the dentate outer molecular layer, the zone containing the lateral perforant pathway synapses. Instead, dysmorphic axons are confined to the corpus callosum just ventral to the site of impact, and occasionally to the alveus, the white matter tract lying along the dorsal surface of the rostral hippocampus.

**Table 2.** Quantitative morphometric analysis of the effects of mTBI on lateral perforant pathway synaptic integrity in the presence of eGFP- or hTauP301L expression. Synaptic density is represented as the mean zinc staining intensity of the injured OML relative to the control OML and determined for each brain section.

Group	N	Injured (%contra +/- sem)	P value (unpaired t-test)
eGFP sham	10	92.8 +/- 2.4	
eGFP mTBI	9	98.8 +/- 7.1	0.4 vs eGFP sham
eGFP mTBI x2	9	95.7 +/- 4.4	0.6 vs eGFP sham
hTau sham	9	91.2 +/- 3.6	0.7 vs eGFP sham
hTau mTBI	6	108.7 +/- 10.7	0.1 vs tau sham; 0.5 vs eGFP mTBI
hTau mTBI x2	9	94.4 +/- 3.6	0.6 vs tau sham; 0.8 vs eGFP mTBI x2

The lack of effect of controlled cortical impact mTBI on integrity of the lateral perforant pathway axons and synapses is not due to failure of the single and double injuries to reach the hippocampus and affect its structure. As described in Section 1 above, there are readily discernable abnormalities in hippocampal structural at 7 days after mTBI in the mouse model of mTBI, in the form of disrupted cytoarchitecture, compression of the dentate gyrus, and scattered acute degeneration of pyramidal neurons.

#### (5) Our methods are capable of detecting perforant pathway neurodegeneration and tau propagation, had they been triggered by mTBI in the acute post-injury period.

To confirm that our analytical methods are capable of detecting partial degeneration of the lateral perforant pathway or trans-synaptic spread of pathological hTau, should they occur after mTBI combined with expression of hTauP301L, we evaluated the integrity of the pathway after administering a dose of AAV-hTau shown previously to induce partial degeneration even in the absence of TBI. All of the experiments described above used intra-entorhinal delivery of 0.5 billion genome copies of the AAV-hTauP301L or AAV-eGFP vectors. This is the maximal dose of the hTau vector that drives robust human tau expression and hyperphosphorylation without causing any loss of perforant pathway neurons or degeneration of axons or synapses. We examined the effects of expressing a higher dose of pathological hTau (0.75 billion genome copies) on perforant pathway integrity and human tau distribution. At this dose, hTau is found not only in the perforant pathway axons of the hippocampal SLM and lateral perforant pathway pre-synaptic terminals of the dentate OML, but its expression expands trans-synaptically to include scattered lateral perforant pathway target neurons, the dentate granule cells in the GCL (Figures 4 and 5, top left). The trans-synaptic transfer of hTau expression in our AAV model occurs only in association with hTau-triggered partial degeneration of the lateral perforant pathway, and is mitigated by a pharmacotherapy that partially blocks the neurotoxicity of hTauP301L (Siman et al., PLoS One 10: e0142340 [2015]).

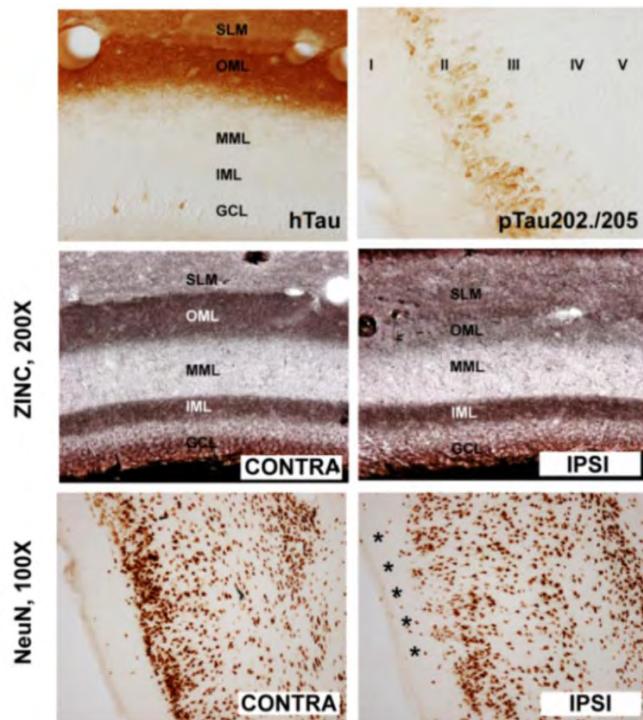


Figure 4. Detection of partial loss of perforant pathway neurons and synapses after delivery of a higher, toxic dose of AAV-hTauP301L.

Top panels – Human tau in dentate gyrus and pTau202/205 in lateral EC layer II 5 weeks after delivery of 0.75 billion genome copies, a dose of hTauP301L that causes partial degeneration of the pathway. 200X mag.

Middle panels – Synaptic zinc staining reveals the hTau-induced partial loss of lateral perforant pathway synapses in the OML (compare the injected with the uninjected side). 200X mag.

Bottom panels - NeuN staining of the lateral EC reveals hTau-driven partial degeneration of layer II neurons of origin for the lateral perforant pathway (just right of the asterisks). 100X mag.

Pathological human tau expression also triggers dose-dependent loss of lateral perforant pathway synapses, as evidenced by a reduced density of zinc staining in the dentate OML, as well as degeneration of lateral perforant pathway neurons, based on a reduced number of NeuN-stained nuclei in layer II of the lateral EC (Figure 4). These results, coupled with our prior findings (J Neuropathol Exp Neurol 72: 1062-1071 [2013]), demonstrate that the zinc and NeuN labeling methods are capable of detecting partial degeneration of lateral perforant pathway axons, synapses, and neurons, had it been triggered by hTau acutely after single or double mTBI.

**(6) Mild TBI does not modify the expression level, subcellular distribution or synaptic spread of human tau or hyperphosphorylated tau in the acute post-injury period.**

With our AAV model for localizing human tau expression to the mouse lateral perforant pathway, we are well positioned to determine whether mTBI exacerbates tau pathology either acutely or chronically after injury. Previously, we obtained evidence that the expression level, cellular and subcellular distribution of total human tau and hyperphosphorylated tau are not altered appreciably by single or double mTBI at 7 days post-injury, and these findings were corroborated over the past year.

Using the HT7 antibody specific for human but not mouse tau, we demonstrated that neither single nor double mTBI change the expression level of human tau, or its cellular and subcellular distribution. After sham injury, single or double mTBI, human tau remained confined to the lateral perforant pathway neurons in entorhinal layer II and the lateral perforant pathway afferents to hippocampal dentate gyrus (e.g., Figure 5, top right). An early step in tau hyperphosphorylation and aggregation is phosphorylation on serine residues 202 and 205, measurable by reactivity with the phospho-specific AT8 antibody. At 5 weeks after unilateral delivery of AAV-hTau and 1 week after sham injury, pTau202/205 accumulates almost exclusively in the perikarya of entorhinal layer II neurons of origin for the lateral perforant pathway (Figure 6). This form of phosphorylated tau is absent from the perforant pathway axons in the hippocampal SLM and terminal field in the dentate OML, as well as from the perforant pathway target neurons, the dentate granule cells (Figures 5,6 bottom). At 7 days after mTBI, there is no appreciable change in either the level, cellular or subcellular distribution of pTau202/205. Thus, as for sham injury, after single or double mTBI neither total human tau nor pTau202/205 is found outside of the lateral perforant pathway neurons, axonal or synaptic fields, and the latter is confined to layer II neurons of the lateral entorhinal cortex (Figures 5,6).

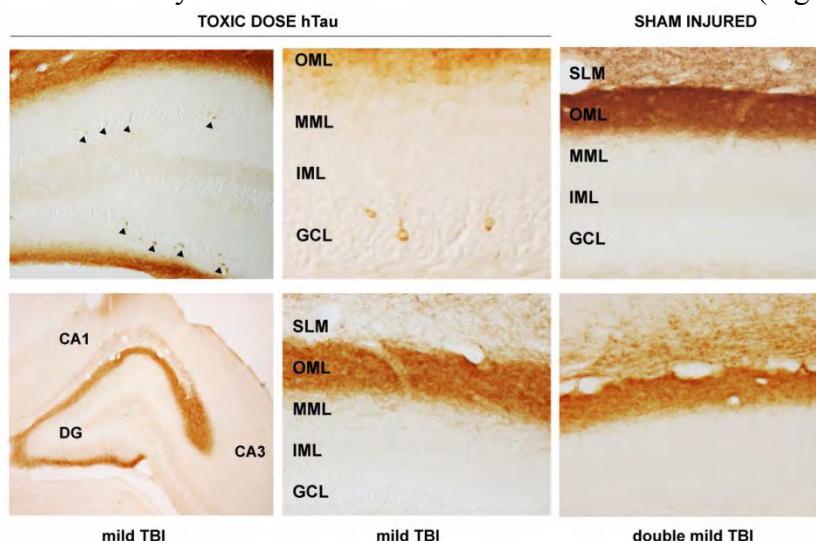


Figure 5. Mild TBI does not cause trans-synaptic spread of total human tau via the perforant pathway acutely post-injury.

Top, left two panels: Total human tau immunostained with the HT7 monoclonal spreads from the lateral perforant pathway afferents (OML) to the dentate granule target neurons (arrowheads) in the granule cell layer (GCL) after delivery of a dose of AAV-hTauP301L that is partially toxic for the pathway. Right panel: In contrast, there is no trans-synaptic spread of human tau after delivery of a non-toxic dose of the tau vector, the dose used in the current study. (See also Siman et al., J Neuropathol Exp Neurol 72: 1062-1071 [2013]).

Bottom panels: Neither single nor double mTBI induce the trans-synaptic spread of total human tau at 7 days post-injury.

In addition to the lack of effect of mTBI on human tau phosphorylation, endogenous mouse tau does not undergo hyperphosphorylation acutely after single or double mTBI. This is manifested by the absence of pTau202/205 in either entorhinal cortex or dentate gyrus at 1 week post-injury in mice expressing eGFP in the lateral perforant pathway (Figure 6, top left panel). Identical findings were observed after immunostaining for a second phosphorylated form of tau, pTau231, using the PHF6 monoclonal. In summary, in the acute post-injury period neither single nor double mTBI changes the phosphorylation of human or endogenous mouse tau, the expression level of human tau, its subcellular compartmentation, or its cellular localization.

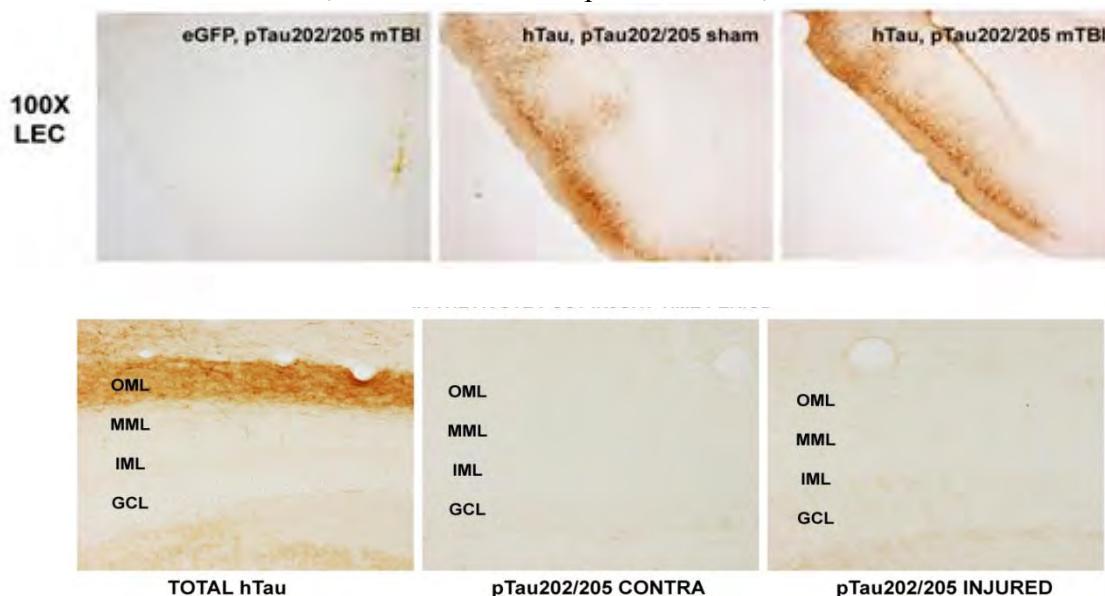


Figure 6. Single and double mTBI do not cause trans-synaptic spread of total or phospho-tau via the perforant pathway or their redistribution within the pathway at 7 days post-injury.

Top panels – Entorhinal cortex: pTau202/205 is not induced in eGFP- or hTau-expressing mice by mTBI.

Bottom panels – Dentate gyrus: pTau202/205 remains excluded from lateral perforant pathway axons and synapses after mTBI.

Bottom left: total human tau is present in the lateral perforant pathway terminal field in the dentate outer molecular layer (OML), but not in perforant pathway target neurons, the dentate granule cells (GCL).

Bottom right two panels: pTau202/205 is not expressed in perforant pathway axons (above the OML) or synapses in the OML after mTBI, identical to cases with sham injury.

**(7) mTBI impairs hippocampus-dependent spatial learning: lack of acute effect of pathological human tau.**

In addition to the array of histopathological methods for evaluating mTBI-induced changes in hippocampal structure following TBI, we completed analysis of hippocampal function this past year, using the Morris Water Maze to assess spatial learning from 3-5 days after single or double mTBI. Our cognitive test data indicate that both single and double mTBI cause subtle impairments in spatial learning at this acute post-injury time period, and unilateral human tau expression in the perforant pathway does not exacerbate the learning deficits, when compared with eGFP expression as a control foreign protein. Additionally, unilateral human tau expression by itself, in the absence of TBI, does not affect spatial learning.

Figure 7 illustrates our data on swim latency to find the hidden platform using visual cues as one of two measures of spatial learning in the mouse. It shows that sham-injured mice expressing either eGFP or hTauP301L learn to locate the hidden platform, based on statistically significant decreases in latency on days 4 and 5 (the second and third days of training) after sham surgery compared with the initial training on day 3. The two genotypes improve equally well. In contrast, eGFP expressing mice subjected to either single or double mTBI exhibited no significant change in swim latency on days 4 compared with day 3, indicating an impaired spatial learning ability. The hTau expressing mice also exhibited injury-induced learning impairment. After single mTBI, they showed longer latencies on days 4 and 5 than on day 3, and the difference at day 5 was statistically significant. Furthermore, after double mTBI, hTau mice showed no improvement in swim latency on days 4 or 5 compared with day 3. Most importantly, there were no significant differences between eGFP- and hTau-expressing mice across any treatment group on any day of training, indicating that unilateral expression of hTauP301L in the lateral perforant pathway does not worsen the mTBI-induced spatial learning deficit acutely post-injury, or cause a learning deficit on its own in the absence of TBI.

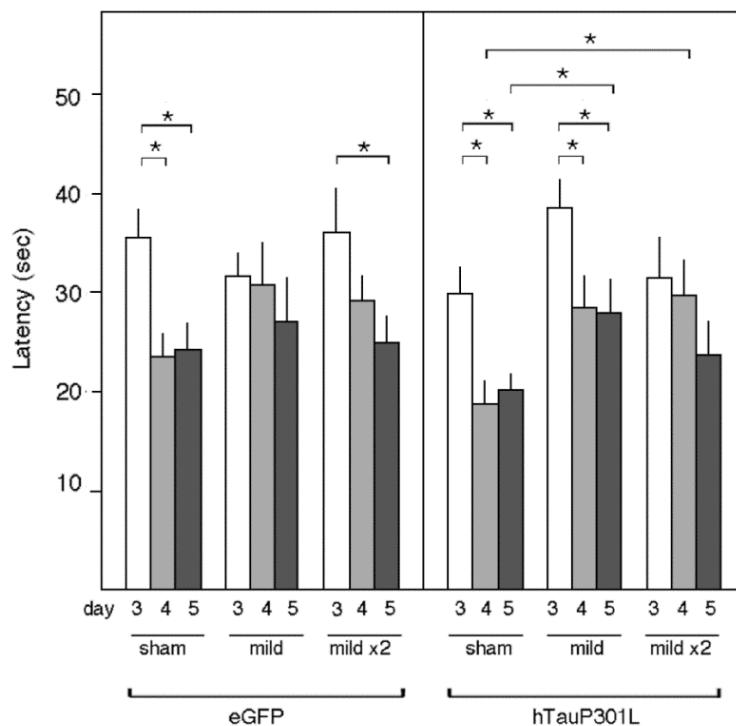


Figure 7. mTBI causes subtle impairment in spatial learning on the Morris Water Maze, an acute effect of injury that is not exacerbated by perforant pathway hTauP301L expression. The mean swim time latency (in seconds+s.e.m.) is shown on three consecutive days of training (6 trials/day) at the indicated days after mice received either sham injury, mTBI, or double mTBI. Both single and double mTBI caused significant learning deficits for the eGFP-expressing mice on day 4, and hTau-expressing mice on day 5. It is noteworthy that there were no significant differences between groups expressing hTauP301L or eGFP in the lateral perforant pathway on any day of training or with any type of injury. \*p<.05, unpaired t-test).

These interpretations of the swim latency data are substantiated by a second measure of spatial learning, conducted using a platform removal trial at the end of training on day 5. Both eGFP- and hTau-expressing mice subjected to mTBI spend less time in the correct quadrant (the one that contained the hidden platform during training) than their respective sham-injured controls, indicating impaired memory for the location of the platform. These spatial learning and memory deficits reached statistical significance for the eGFP single mTBI and the hTau double mTBI groups ( $p < .02$ ). Once again, there were no significant differences in the percent time spent in the correct quadrant after sham injury, mTBI, or double mTBI comparing the eGFP- with the hTau-expressing mice. From these collective data, we conclude that (i) mice exhibit spatial learning with repeated training on consecutive days; (ii) both single and double mTBI cause subtle but discernable impairments in two measures of spatial learning; and (iii) unilateral expression of hTauP301L in the lateral perforant pathway does not worsen the deleterious effects of single or repetitive mTBI on spatial learning.

Given that pathological human tau has no worsening effect in the acute post-injury period on hippocampus-dependent spatial learning, perforant pathway synapse integrity, or survival of the neurons of origin for the pathway (completing milestone 1 of the project), the second phase of our project is well positioned to evaluate any influences of pathological human tau on hippocampus-dependent spatial learning and lateral perforant pathway structural integrity that develop only during the chronic post-TBI period (milestone 2).

#### **(8) Blood-based biomarkers for mild TBI-induced brain damage: Serum SNTF is not elevated significantly at 7 days after single or double mTBI.**

During the past year, we quantified serum levels of a protein biomarker for TBI-induced axonal damage discovered and characterized previously by the Siman laboratory, the calpain-derived  $\alpha$ -spectrin N-terminal fragment SNTF ( $\alpha$ -spectrin 1-1176). SNTF levels rise in the blood on the day of a human concussion treated in the emergency room, and are elevated preferentially in those cases exhibiting white matter abnormalities detected by advanced neuroimaging, as well as persisting cognitive performance problems. In professional athletes, serum SNTF increases after an in-game concussion preferentially in players that will go on to develop persisting post-concussion symptoms affecting their return to play. Histopathological studies demonstrate that this calpain derivative normally is below the limit of detection in the brain, but accumulates within damaged axons after TBI in humans and mTBI in a large animal experimental model of concussion (Johnson et al., *Acta Neuropathol.* **131**, 115 [2016]). On these and other bases, SNTF has emerged as the lead blood biomarker for the prognosis of concussion, and its elevation appears to be a surrogate marker for diffuse axonal injury.

Our biomarker aims in the current study are to (i) determine whether serum SNTF is persistently elevated in a mouse model of mTBI out to 7 days post-injury; and (ii) if so, evaluate the relationship between serum levels of the markers and the measures of brain damage and

behavioral dysfunction after TBI in the presence and absence of hTauP301L expression. To enable this comparative biomarker study, sera were analyzed from mice given comprehensive histological and behavioral evaluations acutely post-injury. Compared with sham injured controls (mean SNTF = 61U +/- 17), neither single mTBI (SNTF = 62U +/- 15) nor double mTBI (SNTF = 59U +/- 19) changed serum SNTF measured at 7 days post-injury. These data are consistent with our longitudinal study of serum SNTF in concussed ice hockey players, where SNTF was elevated from 1-36 hours post-injury, and then declined to pre-season baseline thereafter (Siman et al., J. Neurotrauma 32, 1294 [2015]). It is also consistent with our study of blood SNTF in concussion cases treated in the emergency room, in which plasma SNTF was elevated on the day of concussion but not at 4 days post-injury in the subset of participants exhibiting persisting cognitive performance problems at 3 months post-injury (Siman et al., Front. Neurol. 18, doi: 10.3389/fneur.2013.00190 [2013]).

Given the lack of appreciable change in serum SNTF at 7 days post-injury in the mouse, the second phase of our study is poised to determine whether this biomarker for diffuse axonal injury increases preferentially in the long-term post-injury period as a surrogate marker for a chronic neurodegenerative condition, and whether pathological human tau expression in the perforant pathway worsens any long-term effect of mTBI to elevate this surrogate biomarker.

#### **(9) Summary of conclusions - milestone I:**

*Milestone I: By month 18 (i) determine whether tau worsens hippocampal structure and function in the acute post-TBI time period; (ii) determine whether mTBI worsens incipient tauopathy acutely after injury; (iii) determine whether blood levels of neurodegeneration biomarkers measured at 7 days post-injury correlate with the acute severity of brain damage and cognitive dysfunction after mTBI.*

During the first two years of the project, this milestone has been completed. Collectively, our data indicate that pathological human tau does not appreciably worsen TBI-induced changes in hippocampal structure and cognitive function acutely post-injury. Our data also show that neither single nor double mTBI worsens tau hyperphosphorylation or promotes its anatomical spread at 7 days post-injury.

How does the conclusion, that no discernable interaction occurs in the mouse brain between pathological human tau and single or double mTBI in the acute post-injury period, relate to current literature on the role of tau pathology in human TBI? They are consistent. The histopathological study of post-mortem human brains obtained from a few days to one month after TBI reported no change in neuronal expression or phosphorylation of tau in the sub-acute post-injury time frame (Smith et al., Neuropathol. Appl. Neurbiol. 29, 496 [2003]).

With the completion of its first phase, our project is now well positioned to address in its second phase whether long-term interactions might occur between tau and mTBI that take time to develop chronically post-injury. The collective data from the acute post-injury time period serve as a baseline for evaluating whether hTau and TBI influence one another chronically after brain injury in a manner not observed in the acute post-injury period. From studies of the chronic effects of TBI in humans conducted thus far, there is a large body of evidence that TBI triggers long after injury the development of neocortical and hippocampal tau pathology (reviewed in Nat. Rev. Neurol. 9, 211 [2013]). Consequently, it is during this long-term post-injury period that tau pathology may possibly contribute to evolving brain atrophy and functional decline after injury, and that single or repetitive mTBI may trigger the development of Alzheimer-type tauopathy.

**(9) Chronic interactions between tau and mild TBI in the mouse model of early-stage Alzheimer-type tauopathy (milestone 2).**

During the latter part of year 2, we initiated the second phase of our study, designed to evaluate interactions between tauopathy and mTBI in the long-term (4 months) post-injury period. Thus far, 70 mice have been genetically modified with the AAV vectors (35 expressing eGFP and another 35 hTauP301L) and 60 of these have been subjected at one month after gene delivery to either sham injury, single or double mild TBI (10 per genotype and injury group). Recently, we began analyses at 4 months post-injury of the effects of pathological human tau on the long-term structural, functional, and biomarker outcomes from mTBI, and of long-term effects of mTBI on the severity and spread of tauopathy. In addition, serum is being obtained from the mice at the time of sacrifice at 4 months post-injury for the study of SNTF and potentially other blood biomarkers that may predict the development of a TBI-induced chronic neurodegenerative condition in the long-term post-injury period. Serum SNTF will also serve as a surrogate marker of chronic neurodegeneration in our search for potential interactions between TBI and pathological human tau that may develop only in the long term after mTBI.

From the initial batch of mice evaluated at 4 months post-injury, examples of which are shown in Figures 8 and 9 below, we report the following preliminary histological observations:

(1) Single and double mTBI produce larger cortical lesions and greater hippocampal disruption at 4 months compared with at 7 days post-injury (Figure 8), confirming previous temporal studies by other laboratories of evolving brain atrophy following experimental TBI. The potential effects of pathological human tau on this long-term brain atrophy after mTBI have never been reported before, and are the subject of our current investigation.

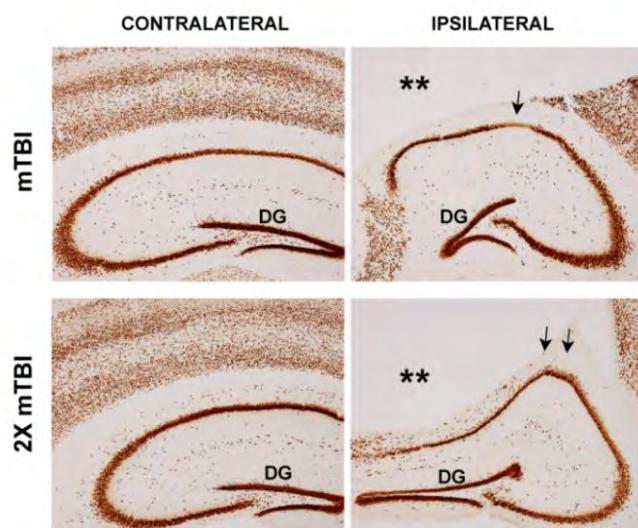


Figure 8. Mild TBI-induced brain damage at 4 months post-injury. NeuN immunostaining of neurons near the site of impact shows the large focal lesions of parietal cortex at 4 months after single or double mTBI (asterisks). Beneath the impact site, the hippocampus is distorted, with disrupted cytoarchitecture in the CA1 sector (arrows), including patches with neuronal loss (single arrow). The dentate gyrus (DG) is markedly compressed and misshapen, as evidenced by alterations in the granule cell layers. The granule cell layer and hippocampal CA3 pyramidal cell layer sometimes show areas of reduced neuronal density. All of these anatomical changes are much more pronounced than at 7 days post-injury.

(2) Expression of eGFP and hTauP301L are sustained in the lateral perforant pathway for 5 months after intra-entorhinal AAV microinjection. Both the hippocampal afferents traversing the hippocampal SLM and their terminal field in the dentate OML continue to exhibit circumscribed foreign protein expression. pTau202/205 labeled with the AT8 antibody is still strongly expressed in and confined to the layer II neurons of origin for the pathway in the lateral entorhinal cortex (Figure 9, top panels).

(3) There is no overt neuronal or synapse loss in the lateral perforant pathway at 4 months post-TBI in mice expressing eGFP in the pathway (Figure 9, middle and bottom panels, left).

(4) There is in our initial analyses no overt synapse or neuronal loss resulting from the sustained expression of a low dose of pathological human tau in the lateral perforant pathway in the absence of TBI (Figure 9, middle and bottom panels, right).

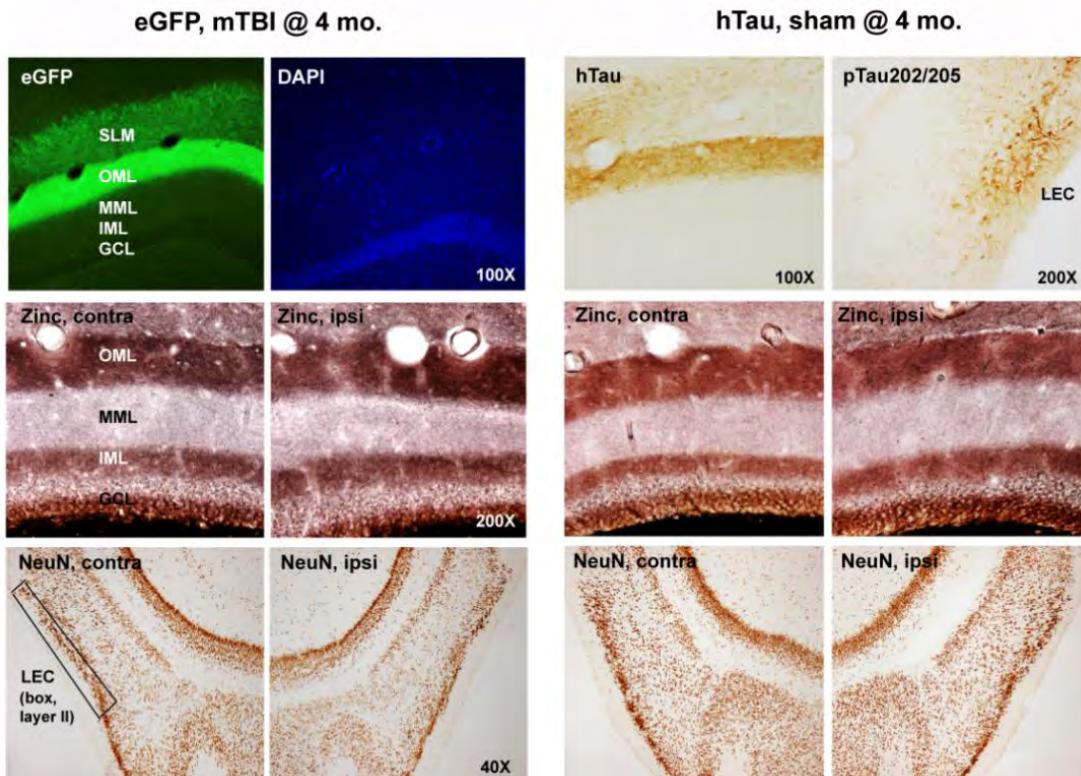


Figure 9. Initial evaluation of transgene expression, synapse and neuronal integrity at 5 months after hTau and eGFP delivery and 4 months after sham surgery or mTBI.

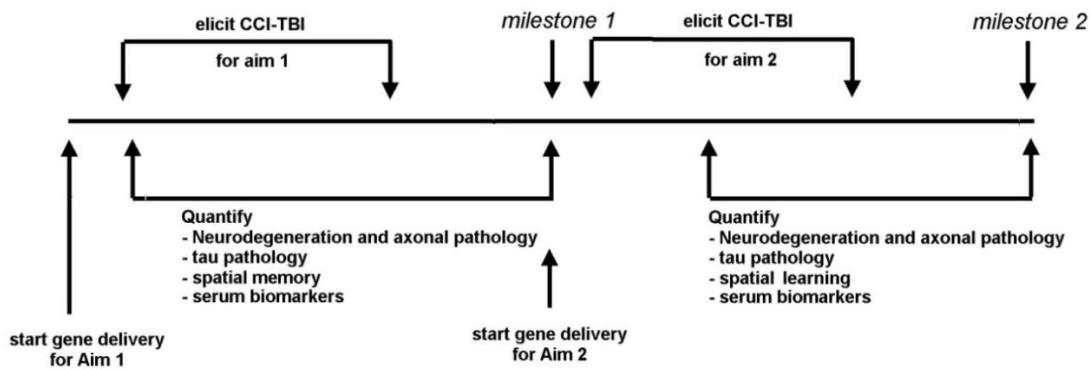
Top panels: Expression of eGFP (left) and hTau (right) are sustained at 5 months after AAV-mediated gene delivery and 4 months after mTBI (coupled with eGFP) or sham surgery (coupled with hTau). Note the robust expression of both proteins in the lateral perforant pathway terminal field in the dentate OML. Also, note that at 5 months after hTau gene delivery, pTau202/205 is abundant in the neurons of origin for the pathway in layer II of the lateral entorhinal cortex (LEC; top right), but undetectable in axons and synaptic terminals of the pathway in the dentate OML, identical to the distribution of pTau acutely after mTBI (data not shown).

Middle panels: Lateral perforant pathway synapses in the dentate OML appear intact at 4 months after single mTBI coupled with eGFP expression, and after sham injury coupled with hTauP301L expression, as evidenced by the persistence of pre-synaptic terminal zinc staining.

Bottom panels: Lateral perforant pathway neurons in the entorhinal cortex appear intact at 4 months after single mTBI, and after 5 months of hTauP301L expression, as evidenced by the persistence of neuronal nuclei in layer II (denoted by the box).

For our study of long-term interactions between pathological tau and mTBI, an additional 55 mice have been genetically modified thus far, and 45 have been subjected at one month after gene delivery to sham injury, single mTBI, or double mTBI. These and additional cases will be used over the coming quarters for characterization of the long-term effects on hippocampal

structure and function of single and double mTBI. In the coming year we will complete milestone 2 and determine whether (i) pathological human tau interacts with mTBI in the long-term post-injury period to worsen chronic outcomes from the injury; (ii) single and repetitive mTBI chronically worsen the severity and spread of an incipient tau pathology; and (iii) serum SNTF exhibits sustained elevation indicative of a developing chronic neurodegenerative condition after mTBI.



## Impact

Mild traumatic brain injury (mTBI) is the most common neurological injury in civilians, and affects over 1.5 million children and adults each year in the United States. Although mTBI is typically undetectable with computed tomography, it can elicit long-term and clinically significant brain dysfunction in ~25% of cases. At the present time, there are neither methods that can identify at an early and treatable stage the subset of mTBI sufferers who will go on to develop acute brain damage and long-term disability, nor clinically proven treatments for improving brain functional outcome. Consequently, new approaches are urgently needed for rapidly identifying mTBI patients in the acute post-injury period who are at risk of suffering persistent brain dysfunction, and for treating these at-risk cases to preserve brain structure and function. Furthermore, accumulating evidence suggests that both single and repetitive TBIs can lead in later life to a chronic, progressive Alzheimer's disease (AD)-like neurodegenerative disorder. New methods are needed to identify those individuals that are beginning to develop TBI-triggered chronic neurodegenerative disease, and new treatments urgently need to be developed for slowing their chronically progressive brain atrophy and cognitive decline.

In the long-term post-injury time period, mTBI shares neuropathological features with AD. Moreover, given that AD is a slowly progressive neurodegenerative and cognitive disorder, and TBI will sometimes lead to chronic progressive brain atrophy and cognitive decline, the question arises whether AD and TBI may share common underlying pathophysiology. One of the pathological hallmarks of AD is the aggregation of the protein tau into neurofibrillary tangles within vulnerable neurons in brain regions important for higher cognitive function. Considerable evidence implicates tau pathology as a key pathogenic driving force for the progressive brain atrophy and inexorable cognitive decline. On the other hand, whereas mTBI will also sometimes cause tau abnormalities that superficially resemble the tauopathy of AD, the pathophysiological roles for tau in the acute and chronic periods after TBI are unknown. Here, we are examining directly and critically whether tauopathy plays important roles in the acute and chronic outcomes from single and repetitive mTBI. Our study evaluates in a well-controlled pre-clinical experimental model the interrelationships between TBI and subsequent AD, thereby fostering discovery of new therapeutic strategies for military personnel, veterans, and civilians exposed to

single or repetitive mTBI. *Based on the histopathological and neurocognitive responses to single and double mTBI observed thus far, our data indicate that the presence of a pathological form of human tau in the mouse perforant pathway projection does not render the neurons, axons, or synapses of the pathway more vulnerable to single mTBI in the acute post-injury period. In addition, a single mTBI does not exacerbate features of tauopathy acutely after mTBI. The third year of the project will extend the study to evaluate potential chronic interactions between tau and mTBI in the long term post-injury time period.*

Finally, the ongoing study is evaluating pre-clinically new diagnostic and prognostic blood tests for identifying at early and treatable stages the subsets of mTBI cases at risk of developing brain damage and long-term dysfunction. Simple blood tests for TBI induced brain damage are vitally needed, and would have major applications for both military and civilian sufferers of mTBI. *Thus far, serum levels of the neurodegeneration biomarker SNTF are not appreciably elevated at 7 days post-TBI. During the final year of the project, serum SNTF will be analyzed as a possible surrogate blood biomarker for chronic neurodegenerative changes induced by mTBI developing only in the long-term post-injury period.*

### **Changes/Problems**

One change was introduced during the second year of the project. Our published sandwich immunoassay method used to quantify serum levels of SNTF was improved by the derivation of a new SNTF-specific monoclonal detecting antibody that yields superior assay performance (higher sensitivity and lower background) than the polyclonal SNTF-specific rabbit serum used previously. This improved next-generation SNTF electrochemiluminescence immunoassay will facilitate our study during the final year of the project of the effects of single and double mTBI, both in the absence and presence of pathological human tau expression in the perforant pathway, on long-term hippocampal structural outcome, using blood SNTF as a surrogate marker for mTBI-induced chronic neurodegeneration.

One problem with biomarker analysis was encountered during the second project year. Whereas the majority of sham-injured mice had serum levels of the neurodegeneration biomarker SNTF below the lower limit of detection when tested at 7 days post-injury, a subset had elevated serum SNTF at this time point. This subset included a small proportion of the sham surgical controls that did not receive any mTBI. We believe this resulted from the double survival surgical procedures for delivering AAV expression vectors and then, one month later, controlled cortical impact mTBI, both of which require craniectomies. We noted that two sequential craniectomies sometimes caused damage to the underlying superficial neocortex, likely leading to an elevation in serum SNTF at 7 days after the second craniectomy unrelated to the mTBI. The elevation in mean serum SNTF for sham-injured mice compared with naïve controls may have masked any effect of the single or double mTBI on the biomarker. This problem will not confound our ongoing study of the effects of human tau and mTBI at 4 months post-injury, since there will be a 4 month delay between the second craniectomy and the serum biomarker evaluation.

### **Products**

Nothing to report.

## **Participants and other support**

1. Dr. Robert Siman  
Role –Principal Investigator  
Effort – 2.4 person months/year  
Contribution – Dr. Siman has directed every aspect of the project. He formulated the experimental strategies. He trained personnel on the requisite methods of stereotaxic neurosurgical viral vector-based gene delivery, animal husbandry, histology, immunohistochemistry, microscopy, quantitative morphometry, and serum preparation. He assisted with histological assessments of perforant pathway neuronal, axonal, and synaptic integrity after traumatic brain injury. He performed photomicroscopic documentation of the research findings thus far, and prepared all quarterly and annual reports. He validated the immunoassays for neurodegeneration biomarkers using new equipment purchased through this award.
2. Dr. Victoria Johnson  
Role – Co-Investigator  
Effort – 1.2 person months/year  
Contribution – Dr. Johnson has performed the controlled cortical impact traumatic brain injuries and sham injuries, and has introduced methodological improvements to enhance the precision and consistency with which the injury device elicits mild TBI. She ensured personnel were trained thoroughly on the evaluation of spatial learning using the Morris Water Maze task. She assisted with histological study of mTBI-induced axonal pathology.
3. Ms. Hongmei Cui  
Role – Research Specialist, Siman laboratory  
Effort – 4.8 person months/year; reduced to 3 person months/year 3  
Contribution – Ms. Cui performed all of the neurosurgical gene delivery, animal husbandry, and histology, and most of the immunohistochemistry evaluating short-term responses to mild TBI. Upon completion of milestone 1, she performed all of the neurosurgical, animal husbandry, and histological methods conducted thus far on the long-term (4 month) outcomes from single and double mTBI. In the coming year, Ms. Cui will take on additional responsibility for conducting immunoassays to measure serum changes in neurodegeneration biomarkers in the long-term post-injury period. She will perform fewer of the histopathological qualitative and quantitative analyses, as some of these responsibilities will be assumed by Mr. Feintech, a recent addition to the Siman laboratory.
4. Ms. Maura Weber  
Role – Research Specialist, Johnson laboratory  
Effort – 4.8 person months/year  
Contribution - Ms. Weber performed the neurobehavioral assessment of hippocampus-dependent spatial learning, using the Morris Water Maze task.
5. Mr. Samuel Feintech  
Role – Research Specialist, Siman laboratory

Effort – 6 person months/year 3

Mr. Feintech is being added to the project to expedite the timely completion by the end of project funding of all quantitative histopathological and blood biomarker analyses of human tau/mTBI interactions in the chronic post-mTBI period. He will conduct histopathological evaluations of tau expression and pathology, neuronal and synapse integrity, and axonal vulnerability of the genetically modified perforant pathway at 4 months after single or repetitive mTBI.

Other support – Over the past year, Dr. Siman received additional grant support as a co-investigator.

“Investigating the neurologic effects of training associated blast”

DARPA award NEU-92-2913

Joshua Duckworth, Principal Investigator

Robert Siman, Co-Investigator (1.8 calendar months effort)

Period: 7/1/16 – 6/30/19

Administration: Henry M. Jackson Foundation

Subaward Specialist: Alison Dineen

Annual direct cost (Siman sub-contract):

Overlap: There is no overlap with the current project. This new award funds a human research study into the effects of heavy weapons training in the military on brain injury and functional status. Dr. Siman is assessing a set of blood biomarkers for neurodegeneration as potential surrogate markers for training-induced brain injury. Longitudinal serum and plasma levels of SNTF, dephosphorylated NFH, and hypophosphorylated NFH are being compared with neuroradiological and neurobehavioral evaluations of training-induced brain structural and functional changes, and with cumulative blast exposures.

### **Special Reporting Requirements**

Not applicable.

### **Appendix**

None to report.